Phage biocontrol to combat Pseudomonas syringae pathogens causing disease in cherry

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Summary

Bacterial canker is a major disease of *Prunus* species, such as cherry (*Prunus avium*). It is caused by *Pseudomonas syringae* pathovars, including *P. syringae* pv. *syringae* (*Pss*) and *P. syringae* pv. *morsprunorum* race 1 (*Psm1*) and race 2 (*Psm2*). Concerns over the environmental impact of, and the development of bacterial resistance to, traditional copper controls calls for new approaches to disease management. Bacteriophage-based biocontrol could provide a sustainable and natural alternative approach to combat bacterial pathogens. Therefore, seventy phages were isolated from soil, leaf and bark of cherry trees in six locations in the south east of England. Subsequently, their host range was assessed against strains of *Pss*, *Psm1* and *Psm2*. While these phages lysed different *Pss*, *Psm* and some other *P. syringae* pathovar isolates, they did not infect beneficial bacteria such as *Pseudomonas fluorescens*. A subset of thirteen phages were further characterized by genome sequencing, revealing five distinct clades in which the phages could be clustered. No known toxins or lysogeny-associated genes could be identified. Using bioassays, selected phages could effectively reduce disease progression *in vivo*, both individually and in cocktails, reinforcing their potential as biocontrol agents in agriculture.

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Introduction

Pseudomonas syringae is a species complex of bacterial plant pathogens that infects over 180 plant species. Many globally important crops fall within the host range of *P. syringae*, making the pathogen one of the most economically destructive (Xin *et al.*, 2018). *Prunus* species, including cherry (*Prunus avium*), are highly susceptible to *P. syringae*, with infection leading to bacterial canker and devastating impacts on commercial fruit production (Hinrichs-Berger, 2004; Hulin *et al.*, 2018a,b). Fruits, leaves and blossoms are susceptible all year. Infection often occurs through leaf scars and wounds in the winter, with symptoms of canker not becoming visible until spring. Large dark wounds appear on the trunk and branches of infected trees, discharging large volumes of gummy sap. Leaves also take on a shot-hole appearance, with the tree's growth compromised, eventually leading to plant death (Hulin *et al.*, 2018a,b). *Prunus* species are infected by the *P. syringae* pathovars *syringae* and *morsprunorum* (Hulin *et al.*, 2018a,b). To date, three pathovars of *P. syringae* have been identified as the main causal agents in bacterial canker of cherry: *P. syringae* pv. s*yringae* (*Pss*), *P. syringae* pv. *morsprunorum* race 1 (*Psm1*) and *P. syringae* pv. *morsprunorum* race 2 (*Psm2*; Hulin *et al.*, 2018a,b). *Pss* has the broadest host range, infecting numerous species of plant, whereas both races of pv. *morsprunorum* are highly specific to *Prunus* species (Bultreys and Kałużna, 2010). *Psm1* and *Psm2* were initially thought to be closely related, based on morphology and virulence towards *Prunus*, though it has since been found that these two pathovars are only distantly related within the *P. syringae* species complex (Freigoun and Crosse, 1975; Hulin *et al.*, 2018a,b).

Bacterial canker of *Prunus* is a difficult disease to control due to high rates of mutation, horizontal gene transfer and species-specific variability (Jones *et al.*, 2007). No specific chemical controls have currently been developed, and resistance to generic chemical controls can emerge and spread rapidly through

populations (Stone and Baker, 2010). Traditional methods for the control of bacterial canker of *Prunus*, such as copper-based bactericides, are increasingly less popular as bacteria develop copper resistance and the environmental damage of copper accumulation has become apparent (Sundin and Bender, 1993; Jones *et al*., 2007). As such, their use is restricted across Europe (Stone and Baker, 2010). Breeding for resistance is one alternative potential route to control the disease but has proved to be challenging and complex as it may need screening for all three pathogens (Farhadfar *et al*., 2016; Hulin *et al*., 2018a,b). Also, the pathogens are varied in aggressiveness and therefore may trigger differing host resistance mechanisms in cherry (Hulin *et al*., 2018a,b).

One potential alternative to control bacterial diseases is through bacteriophage biocontrol. Bacteriophages (phages) are viruses that infect bacteria, using the bacterium's metabolism to complete their replication, resulting in the lysis of their bacterial host, without directly causing harm to plants or animals. Phages are one of the most abundant types of organisms in the biosphere and are almost always highly specific to their target bacterial host. The potential of phage against *Pseudomonas syringae* pathovars *actinidiae* and *aesculi* have been explored as potential biocontrol agents (Di Lallo *et al*., 2014; James, 2015). The high degree of host specificity, the lytic life cycle, the ability to persist and proliferate in the environment makes bacteriophage potential candidates for the treatment of bacterial infections in both humans and plants (Buttimer *et al*., 2017). The use of phage as biological control agents requires knowledge of the host specificity and an understanding of the way in which the host bacterium and phage interact, tailored towards a specific pathogen. Here, we isolated, identified and characterized phages that can infect *Pss*, *Psm1* and *Psm2*. The efficacy of the phage, both individually and in cocktails, to reduce disease progression *in vivo* were investigated to understand the potential for practical use of these phages as biocontrol agents.

Results

A large variety of phages were isolated from cherry tree leaves, bark and soil samples

To obtain a range of phage isolates, sampling was done at six different geographical sites. A total of 60 trees were sampled for testing, taking leaves and soils from all sites. Bark was only sampled from 20 trees in the NIAB EMR cherry trial orchard, but not from commercial cherry trees, due to restrictions. All 60 samples (60 soil, 60 leaf and 20 bark samples) were processed to obtain suspensions, which were plated onto bacterial lawns of *Pss*, *Psm*1 and *Psm2* for the production of plaques. In total, 70 phage isolates were obtained (Table S1), 60 from all 60 soil samples, one from the leaf samples and nine from the bark samples. 45% of the phages were collected from the commercial cherry orchard, 26% from the NIAB EMR trial plots, 16% from the old cherry orchard in Sittingbourne, 7% from the old cherry orchard in Milstead, 4% from the Brogdale National Fruit Collection and 2% from the young cherry orchard in Milstead. All phages produced clear plaques and plaque sizes varied from less than 1–10 mm in diameter with 57% being large (8–10 mm), 22% medium (5–7 mm), 20% small (2–4 mm) and 1% very small (< 2 mm; Table S1).

Table 1. Host range analysis of 18 phages isolated from soil, leaf and bark of *Prunus avium*.

Phage name	Sample type	Phage size	Family	PSS (9097)	PSM1 (5244)	PSM2 (5255)	Pss 9293	Pss 9630	Pss 9644	Pss 9654	Pss 9659	Psm1 5300	Psm1 9326
MR1	Soil/[a]D	Large	*Podoviridae*	✓	✓	✓		✓	✓		✓	✓	✓
MR2	Soil	Large	*Podoviridae*	✓	✓			✓	✓		✓	✓	✓
MR3	Soil	Medium	*Podoviridae*	✓	✓	✓		✓	✓		✓	✓	✓
MR4	Soil	Medium	*Podoviridae*	✓			✓	✓	✓			✓	✓
MR5	Soil	Large	*Podoviridae*	✓			✓	✓	✓		✓		✓
MR6	Soil	Large	*Podoviridae*	✓				✓	✓			✓	✓
MR7	Soil	Large	*Podoviridae*	✓			✓	✓	✓	✓	✓	✓	✓
MR8	Bark	small	*Podoviridae*	✓			✓	✓	✓		✓	✓	✓
MR9	Soil	large	*Podoviridae*	✓				✓	✓	✓	✓	✓	✓
MR10	Soil	large	*Podoviridae*	✓				✓	✓	✓	✓	✓	✓
MR11	Soil	large	*Podoviridae*	✓				✓	✓	✓	✓		✓
MR12	Leaf	Large	*Podoviridae*	✓			✓	✓		✓	✓	✓	
MR13	Soil	Small	*Siphoviridae*	✓	✓	✓		✓		✓		✓	✓
MR14	Soil	Small	*Myoviridae*	✓	✓	✓		✓					
MR15	Soil	Medium	*Siphoviridae*	✓	✓	✓		✓		✓		✓	✓
MR16	Soil	Medium	*Podoviridae*	✓	✓	✓		✓			✓		
MR17	Soil	Large	*Podoviridae*	✓				✓					
MR18	Soil	Small	*Podoviridae*	✓				✓					

Pss, *Pseudomonas syringae pv. syringae*; Psm, *P. syringae* pv. *morsprunorum*; Ps, *Pseudomonas syringae*. *Pss* 9097, *Psm1* 5244 and *Psm2* 5255 were used as bacterial hosts to isolate phage from environmental samples. 13 highlighted phages were selected for further study. Phage size: Large (8–10 mm), medium (5–7 mm) and small (2–4 mm).
a. Phage MR1 was collected from a disease (D) tree.

Host range enabled a first microbiological selection of relevant phage

For use of phage in biocontrol, host specificity of the phage is an important factor to consider. To test the host range of the phage isolates, a range of different bacteria were tested for susceptibility. The phage exhibited a differential ability to infect and lyse different *Pss* and *Psm* isolates as well as other pathogenic *Pseudomonas syringae* pathovars including *P. syringae* pv. *tomato* DC3000, *P. syringae* pv. lachrymans 789 and *P. cichorii* 907. However, the phages were unable to infect beneficial bacteria such as *Pseudomonas fluorescens* ATCC 17400, *P. fluorescens* F113, *P. poae* PpR24, *P. fluorescens* PFR37, *P. putida* PaW340, *P. fluorescens* Pf-5, *P. fluorescens* Pf-01, *P. fluorescens* WCS 365 (Table 1).

A subset of 18 of the phages were selected based on their complementary host range and further characterized. Transmission Electron Microscopy was employed to image the morphology of the phage and estimate their size (Fig. 1). All 18 phages exhibited typical head and tail morphologies associated with the order of the *Caudovirales*. Most phages were podoviruses, while phage MR14 was a myovirus and phages MR13 and MR15 showed a siphovirus morphology.

RAPD-PCR was used to ascertain the level of genetic diversity, to compare banding patterns for all 18 phages and to subselect most diverse phages. Phage DNA was extracted, and amplicons generated using a single decamer primer, P1, which binds within the phage genome and amplifies random regions. The fragments were then separated by size using gel electrophoresis, producing distinctive band patterns that could be used to compare the different phage (Fig. S1). Banding patterns were unique for most phage samples. Based on RAPD-PCR banding patterns, 13 phages showing maximum variation were chosen for further study (Table 1).

Killing curves of relevant Pss strains indicated a high efficacy of the selected phages

To examine the dynamics of phage virulence, killing curves were generated by infecting exponentially growing cultures of *Pss, Psm1 and Psm2* for the 13 individual phages and two phage cocktails, at different multiplicity of infections (MOI; Fig. 2 and Fig. S2). The optical density of the bacterial cultures alone at 600 nm reached 0.7 over the 19 h period. All phage tested were effective at reducing the OD of the bacterial host, but there were diverse differences in the dynamics of bacterial killing by different groups of phages. In all cases, in the presence of the phage, bacterial OD decreased almost immediately. Some phages (MR6, MR7, MR15, MR16 and MR18) caused a particularly rapid decrease in the bacterial population. In all cases, including the use of the two cocktails, increases in OD were observed after 15–19 h in the broth cultures, suggesting the emergence of resistant bacteria within the population.

Different concentrations of phage were observed to have different effects on the bacterial populations. The higher the titre of the phage, the greater their ability to decrease the growth of the bacterial host and in less time. The lowest titres were consistently slower at reducing OD levels. However, phages MR13 and MR14 displayed a modest killing effect with the two lowest MOI while the two highest MOI exhibited delayed killing effects. The phage that appeared most effective over

Psm1 9629	Psm1 9646	Psm1 9657	Psm2 5260	Psm2 sc214	Psm2 PSMR2 leaf	Psm2 9095	Ps 9654	Ps 9643	*P. s. tomato* DC3000	*P. s. lachrymans* 789	*P. cichoriri* 907
✓	✓	✓	✓	✓	✓	✓	✓	✓	✓		✓
✓	✓	✓	✓	✓	✓	✓		✓	✓	✓	✓
✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
✓		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
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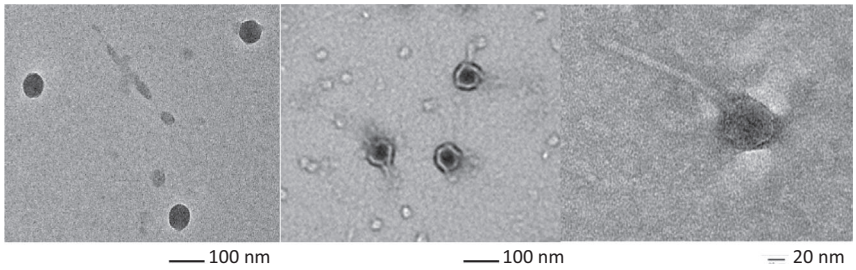


Fig. 1. Morphology of phage MR2, MR14 and MR15 determined by Transmission Electron Microscopy (TEM). These phages belong to *Podoviridae*, *Myoviridae* and *Siphoviridae* respectively.

time, with less resistance emerging, were MR15, MR16 and MR18. At high titre (MOI 10 and MOI 1), phage MR13 and MR14 reduced bacterial OD effectively, with the emergence of resistance (an increase in broth optical density) appearing after 19 h. Bacterial resistance to all the phages (except MR13, MR14, MR15 and MR18) was observed in each bacterial host population at higher titres (MOI 10 and MOI 1) after around 15 h of incubation. However, despite the growth, the bacterial OD was considerably lower (0.2–0.3) compared to the bacteria alone control (OD 0.7) at 19 h. At the highest titre (MOI 10), the bacterial host populations developed resistance to both cocktails after around 15 h. However, at lower titres (MOI 1, 0.1 and 0.01), low levels of bacterial resistance only emerged at around 19 h.

Assessing the temperature sensitivity of the phage collection

A key consideration for phage use in biocontrol is storage and efficacy after storage. To this end, we investigated the effect of temperature on the viability, and thus stability, of the 13 phages in collection (Fig. 3 and Fig. S3). This was tested by storing the phage in phage buffer at different temperatures for eight weeks and performing plaque assays using different dilution series each week. This was then repeated after six months. The phages were stable in phage buffer at all temperatures, but at 37°C, the phage viability was reduced by about tenfold after week six and almost all phages lost their viability after six months stored in this temperature. The results showed that all phages were stable and viable at −20°C ($P < 0.0001$), 4°C (P: 0.02) and 20°C ($P < 0.0001$) after six months. However, −20°C remains the best temperature to store the phages, as not only did they maintain their concentration and viability after 6 months, but they also produced clear plaques with no bacterial resistance emerging. Phage MR1, MR2 and MR7 ($P < 0.0001$) were the most stable at 20°C. The lower the titre of the phage at the start, the less they lost their viability over time. Phage MR1, MR8, MR13 and MR16 starting titre was 10^4 pfu per ml. These phages lost their titre by almost tenfold by 6 months compared to other phages where the starting titre was 10^5–10^6 pfu

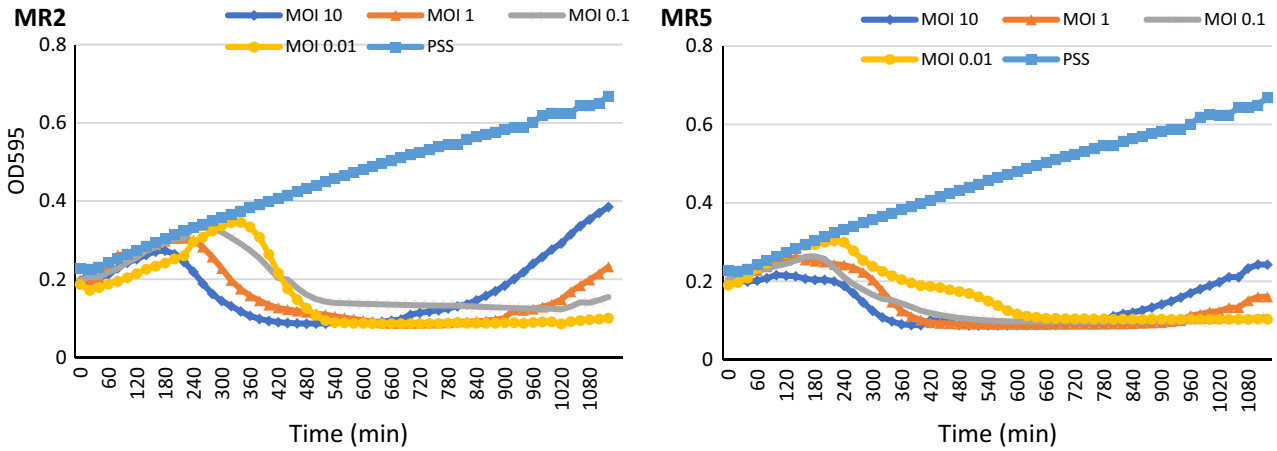


Fig. 2. In vitro killing curve displaying the effect of two phages used in this study on *Pseudomonas syringae* pv. *syringae* (*Pss*) population growth. To ease visualization, each individual killing curve is shown at different multiplicity of infection (MOI) of 10, 1, 0.1 and 0.01, against the bacteria-only control. The bacteria-only control is the same in every graph. Values are average of two replicates.

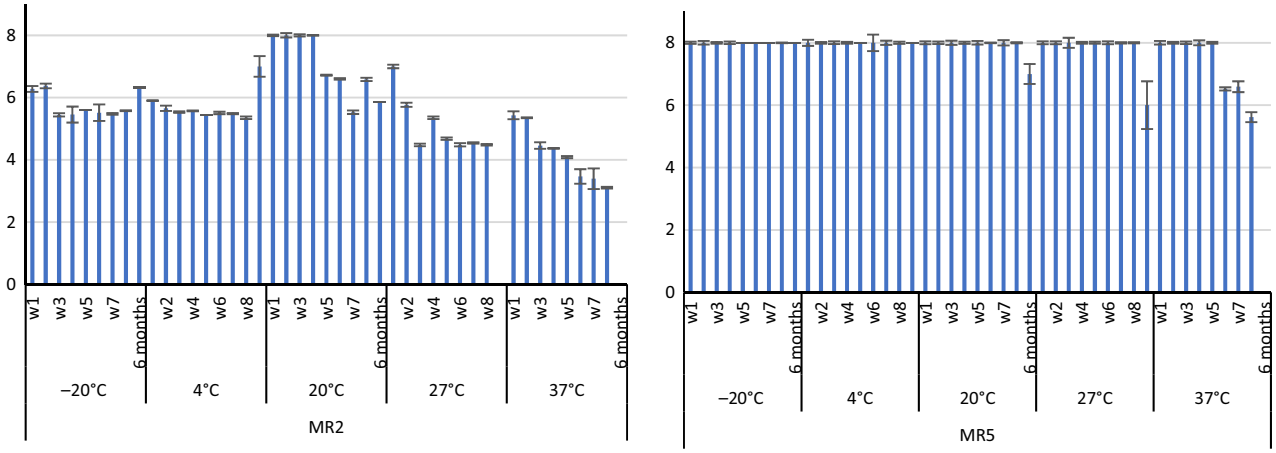


Fig. 3. Temperature stability of two phages used in this study. Each value is a mean of nine replicates, and error bars represent the standard error of the mean (df: 1168, s.e.d: 0.2 (phage*temperature*time)).

per ml. The higher titre phage reduced almost 100-fold after six months.

Phage MR4, MR5, MR6 and MR12 ($P < 0.0001$) were the optimal phages in terms of survival and viability at all temperatures, with phage MR5 (P: 0.0004), MR7 ($P < 0.0001$), MR8 (P: 0.0002) and MR12 (P: 0.03) being the only viable phages at 27°C after 6 months. The larger the phage plaque size, the better they survived at different temperatures. Small plaque size phages (MR8, MR13 and MR14) lost their viability faster compared to the large and medium plaque size phages.

Phage genome sequencing revealed specific phage clades and allowed further selection based on absence of virulence and lysogeny-associated phage genes

To further differentiate between all 13 phages and investigate how closely they are related, genomic DNA was extracted and genome sequencing was performed. The genomes were sequenced ranging between 39 074 bp and 51 347 bp (Table 2). ORFs were identified, and similarity at the protein level was verified by sequence similarity analysis. Where we found more than 65% nucleotide sequence similarity to other existing phage within the database, we used that genome as a reference for annotation. BLASTn comparison of the 13 phages based on their genome sequences confirms the subdivision of the phages into five distinct phage clades (Table 2, Fig. 4).

Phage MR1 and MR2 had 90% similarity to *Pseudomonas* phage PPPL-1 (accession number: NC_028661). Phage PPPL-1, a podovirus, has a lytic activity against *Pseudomonas syringae* pv. *actinidiae*, the causal agent of kiwifruit bacterial canker. Phage MR4 had 89% homology to *Escherichia* phage ECBP5 (accession number: NC_027330). Phage ECBP5 belongs to the *Podoviridae*, which infects avian pathogenic *Escherichia coli* (APEC). Phage MR5, MR6, MR7, MR8, MR12, MR16 and MR18 had genome wide homology between each other, but only less than 50% similarity to *Pseudomonas* phage phi-2 (accession number: NC_013638). Therefore, these seven phages can

Table 2. Whole genome sequence characteristics of 13 phages used in this study.

Phage	GenBank accession number	Genome length (bp)	%G + C	Terminators	ORFs	Bacterial host
MR1	MT104465	39 033	56.2	7	82	*Pss* strain 9097
MR2	MT104466	40 986	57.6	11	139	*Pss* strain 9097
MR4	MT104467	44 816	45.7	11	101	*Pss* strain 9097
MR5	MT104468	41 314	57.6	6	130	*Pss* strain 9097
MR6	MT104469	41 300	57.6	6	129	*Pss* strain 9097
MR7	MT104470	41 705	57.5	6	118	*Pss* strain 9097
MR8	MT104471	41 371	57.5	4	107	*Pss* strain 9097
MR12	MT104472	41 368	57.5	4	109	*Pss* strain 9097
MR13	MT104473	47 456	57.9	27	159	*Psm1* strain 5244
MR14	MT104474	46 593	58.7	17	132	*Psm1* strain 5244
MR15	MT104475	51 347	58.1	28	163	*Psm1* strain 5244
MR16	MT104476	42 767	57.6	5	128	*Pss* strain 9097
MR18	MT104477	41 420	57.5	6	128	*Pss* strain 9097

be considered as a novel species based on current ICTV guidelines (Adriaenssens and Brister, 2017). Phage phi-2 (accession number: FN594518) belongs to the *Podoviridae* family and infects *Pseudomonas fluorescens* SBW25. Phage MR14 had only 2% sequence similarity to *Yersinia* phage fEV-1 (accession number: LT992259) and was also considered a novel phage. Phage MR13 and MR15 had 65% similarity to *Pseudomonas* phage phiPSA1 (accession number: NC_024365). Phage phiPSA1, a member of the *Siphoviridae* family, is a temperate phage, infecting *P. syringae* pv. *actinidiae*.

All isolated podoviruses encoded a DNA and RNA polymerase, primase, ligase, endo- and exo-nuclease, collar and head-tail connector, structural, capsid and tubular protein, internal virion protein and terminase. No toxin genes and bacterial virulence genes were discovered in the phage genomes, which supports their suitability for phage biocontrol. No genes related to lysogeny were discovered in the phage genomes, except for phage MR13 and MR15. These two phages had the Cro/CI family transcriptional regulator. The Cro repressor turns off early gene transcription during the lytic cycle and CI repressor is required to maintain lysogenic growth (Ohlendorf *et al*., 1998). Both phages had one tRNA, with predicted proteins for a phage integrase as well as a putative repressor. The presence of these genes confirms that these two phages are probably temperate phage. Genome sequences of all phages were submitted to the GenBank database, and the accession numbers are provided in Table 2.

The genome sequence confirms the TEM results that all 13 phages belong to the *Caudovirales* order, with typical head and tail structure observed for myo-, podo- and siphoviruses.

Bacterial resistance development allowed assessment of cross-resistance between phages

A bacterium can develop resistance to a phage due to different mechanisms. Therefore, understanding phage resistance is an important factor when choosing a phage as a biocontrol agent. This was tested by isolating spontaneous bacteriophage-insensitive mutants (BIMs) of *Pss* for all 13 phages used in this study. Phage and bacteria were plated at MOI 1, and after 48 h of incubation at 27°C, emerging colonies were counted and about four colonies were cultured and streaked to single colonies at least twice to ensure that a single clonal isolate was selected and to remove any phage particles. The *Pss* BIMs frequency was observed as follows: phage MR1 and MR2, MR4, MR7 and MR18 (2×10^{-7}), MR5 (3×10^{-7}), MR8 (7×10^{-7}), MR12 and MR13 (3×10^{-7}), MR14 (3×10^{-7}), MR15, MR16 and cocktail7 (1.5×10^{-6}), and cocktail13 (8×10^{-7}). To validate the identity of the BIMs and exclude the possibility of contaminations, at least one BIM for each phage was analysed with the rep-PCR. The resulting BIMs banding patterns were compared to the original *Pss*, with all the BIMs appearing identical to the wild-type *Pss* (Fig. S4). A second plaque assay was performed to examine if each wild-type phage was able to lyse its corresponding BIM, using a single purified BIM isolate. Only phage MR7 (9×10^{-2} pfu ml^{-1}), MR12 (5×10^{-3} pfu ml^{-1}) and MR13 (3×10^{-3} pfu ml^{-1}) were able to lyse their corresponding BIMs, however, with lower efficiency.

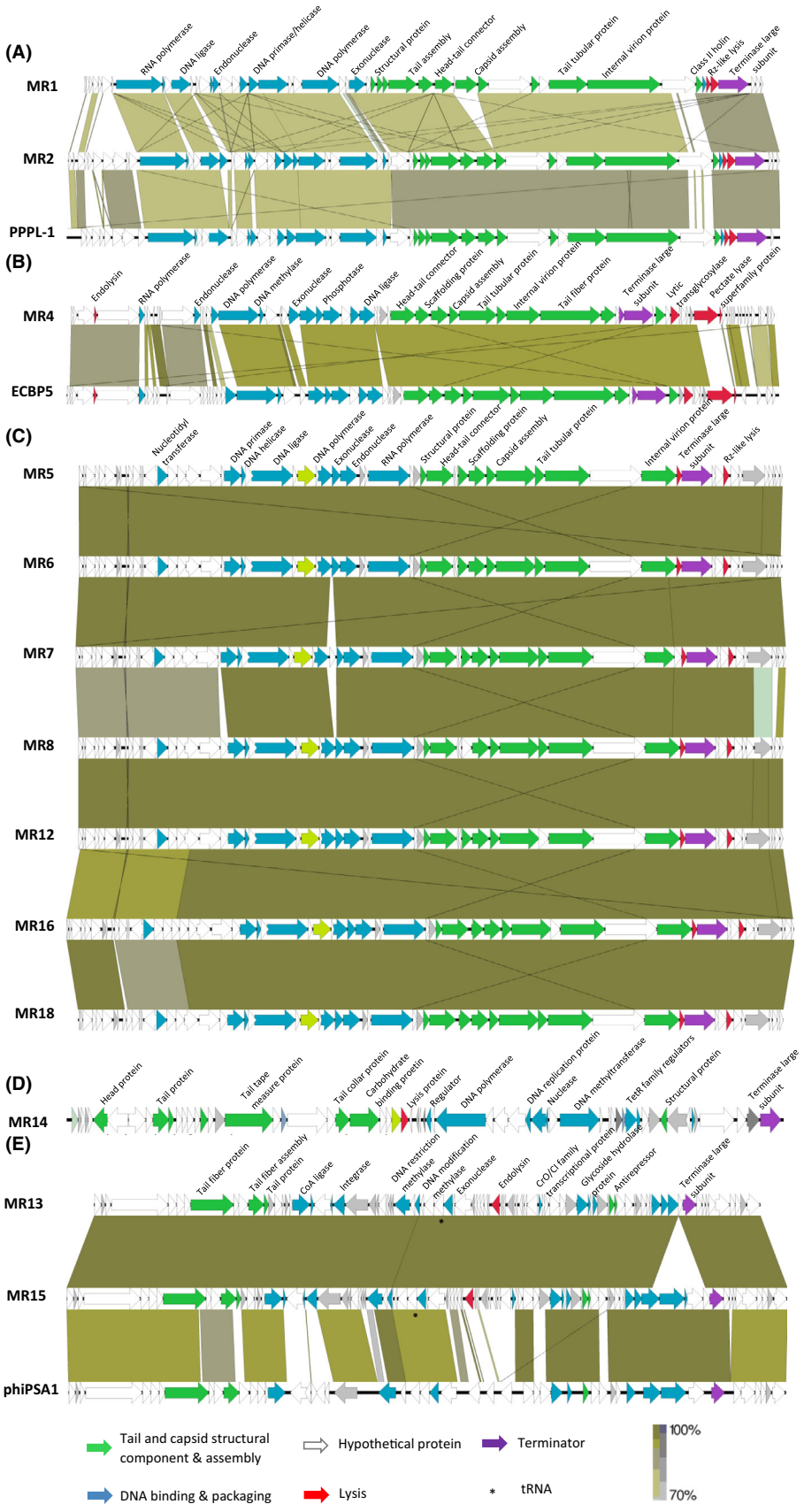
All 13 phages were tested against each BIM to examine whether cross-resistance occurred (e.g. BIM1, generated after co-incubation with phage MR1 was tested against the other 12 phages and both phage cocktails). Some bacteria have developed resistance not only to the phage that generated them but also to other phages. All phages were able to lyse the resistant bacteria generated using phage MR7, MR12 and MR13.

All the BIMs were tested for the spontaneous release of phage particles, and no plaque was observed suggesting there was no induction of lysogens although this does not preclude the possibility that phage progeny could be produced under the conditions used. All 13 phages isolated in this study produced clear plaques, which might indicate their lytic lifecycle, even though phage MR13 and MR15 that could potentially be temperate phages based on their genome sequences.

In planta *bioassay proved the phage-biocontrol cocktail significantly reduces bacterial infections*

Another essential consideration for phage use in biocontrol is to examine their infectivity *in planta*. To test if the phages were effective in a model system, an established bean infection system was used. *In vivo* inoculations of bean plants were performed to investigate whether the

Fig. 4. BLASTn comparison of 13 phages using Easyfig (Sullivan *et al*., 2011). The comparison was performed with BLAST 2.2.28, and the regions of similarity greater than 70% are shown by green shading.
A. Phage MR1 and MR2 aligned with reference genome *Pseudomonas* phage PPPL-1.
B. Phage MR4 aligned with reference genome *Escherichia* phage ECBP5.
C. Phage MR5, MR6, MR7, MR8, MR12, MR16, MR18.
D. Phage MR14.
E. Phage MR13 and MR15 aligned with reference genome *Pseudomonas* phage phiPSA1.



phages were capable of reducing *Pss* populations in a plant environment. Bean leaves were sprayed with *Pss* strain 9097 at the concentration of 10^8 cfu ml^{-1} either alone (control) or with phage at 10^6 pfu ml^{-1}. All 13 phages alone and both cocktails were used, and treatments were replicated five times. Leaves were collected at 0 h after inoculation and then every 24 h for 5 days, and the number of bacterial colonies was counted. At time zero (essentially 1 h from spraying to sampling and plating), phage presence in many cases instantly reduced the *Pss* population by at least 15%–40%. Phage MR6 (P: 0.0001) and MR7 ($P < 0.0001$) reduced the cfu per ml of the bacterial population by 50%, by the end of the experiment, compared to the non-phage treated leaves (Fig. 5). The cocktail of all 13 phages ($P < 0.0001$) and cocktail7 ($P < 0.0001$) reduced the *Pss* population cfu ml^{-1} 15% throughout the experiments. There was a reduction of 20% in cfu per ml of bacteria for phage MR1 (P: 0.0001), MR4 (P: 0.0001), MR8 (P: 0.05), MR12 (P: 0.0004), MR13 (P: 0.06), MR15 (P: 0.003), MR16 (P: 0.005) and MR18 (P: 0.002) compared to the control by 96 h after inoculation. Phage MR2 and MR5 caused a 10%–30% reduction in cfu per ml of bacteria by 48 h after inoculation, but no reduction, thereafter, compared to the control.

Given the simple model system appeared to provide useful insights to the effects of the phages, the approach was then trialled with cherry. Cherry leaves were sprayed with *Pss* strain 9097, *Psm1* strain 5244 and *Psm2* strain 5255 at the concentration of 10^8 cfu ml^{-1} either alone (control) or with phage at 10^6 pfu ml^{-1}. Phage MR6, MR8, MR15 and MR16 individually and cocktail13 and cocktail7 were used. These individual phages were chosen based on the bean experiment results. Cherry leaves were collected two days after the initial bacterial inoculation (week zero) and then every week for 5 weeks, and the number of bacterial colonies was counted. Phage MR6 ($P < 0.0001$) and MR15 ($P < 0.0001$) reduced the *Pss* population by almost 80% and 100% at time zero (week zero), respectively, and maintained the population at low level. However, the *Pss* population increased at week five, but it was still 30% lower than *Pss* alone (control) population. MR8 reduced the bacterial population (cfu per ml) by week two by almost 80%, and however, at week five, it reduced the *Pss* population by 20%. MR16 (P: 0.06) reduced the bacterial population to almost zero by week five. The two cocktails suppressed *Pss* population growth to a low level until week five after which the population increased, although overall it was still 50% less than *Pss* alone population (Fig. 6). *Psm1* and *Psm2* did not infect the leaves as well as Pss, so data were not presented here.

Finally, cherry twig inoculations were performed in October 2019 using *Pss*, *Psm1* and *Psm2* and delivering the same phages as above. Samples were taken 40 days after inoculation. All phages individually and both cocktails could reduce the population of all three bacteria. MR6 ($P < 0.0001$) reduced *Pss* and *Psm1* population by almost 20% and *Psm2* population by 40%. There was a 60% reduction in the cfu per ml of all three bacteria by phage MR8 ($P < 0.0001$). Phage MR15 ($P < 0.0001$) reduced population of *Pss* by 30% and *Psm1* and *Psm2* by 40%. Phage MR16 (P: 0.002) reduced population of *Pss*, Psm1 and Psm2 by 15%, 30% and 45% respectively. Cocktail13 ($P < 0.0001$) reduced cfu per ml of *Pss*, *Psm1* and *Psm2* by almost 10%. There was a 30% reduction in the population of all three bacteria by cocktail7 ($P < 0.0001$; Fig. 7).

Discussion

Bacterial canker of *Prunus* is an annually occurring problem for the cherry fruit industry globally, causing up to 75% loss of trees (Spotts *et al*., 2010; Hulin *et al*., 2018a,b). Three pathovars of *Pseudomonas syringae*: *Pss*, *Psm1* and *Psm2* have been identified as the main causal agents of bacterial canker of cherry (Li *et al*., 2015). In this study, we have identified a number of phages that have the ability to infect and lyse these bacterial pathogens and therefore have the potential to be used as biological control agents of bacterial canker of cherry trees. For a phage to be an effective biological control agent, it is preferable that it has the ability to lyse a range of genetically diverse strains of a bacterial pathogen. All phages used in this study were originally isolated using *Pss* 9097, *Psm1* 5244 and *Psm2* 5255, but almost all phages were equally able to lyse *Pss* 9630, *Psm1* 9646 and *Psm2* 5260, which are closely related strains (Hulin *et al*., 2018a,b). The host range of the phages isolated in this study showed they are entirely specific to *Pseudomonas syringae*, with no detrimental effects to other, beneficial bacteria. Determining the host range of each of the phages enabled the selection of cocktails capable of infecting the widest variation of related bacterial pathogens. All phages, including temperate phage MR13 and MR15, produced clear plaques, displaying a lytic infection cycle, which limits the risk of horizontal gene transfer of pathogenicity possible with lysogenic phage (Penades *et al*., 2015).

To use phage as biological control agents, the phage must be able to persist in the phyllosphere, under variable conditions. Results from the temperature stability assay showed that all 13 selected phages were capable of surviving in different environmental temperatures typical of the annual weather variations in the UK. The phages were stable and were able to lyse bacterial strains after eight weeks of incubation at temperatures ranging from −20°C to 37°C. After six months of

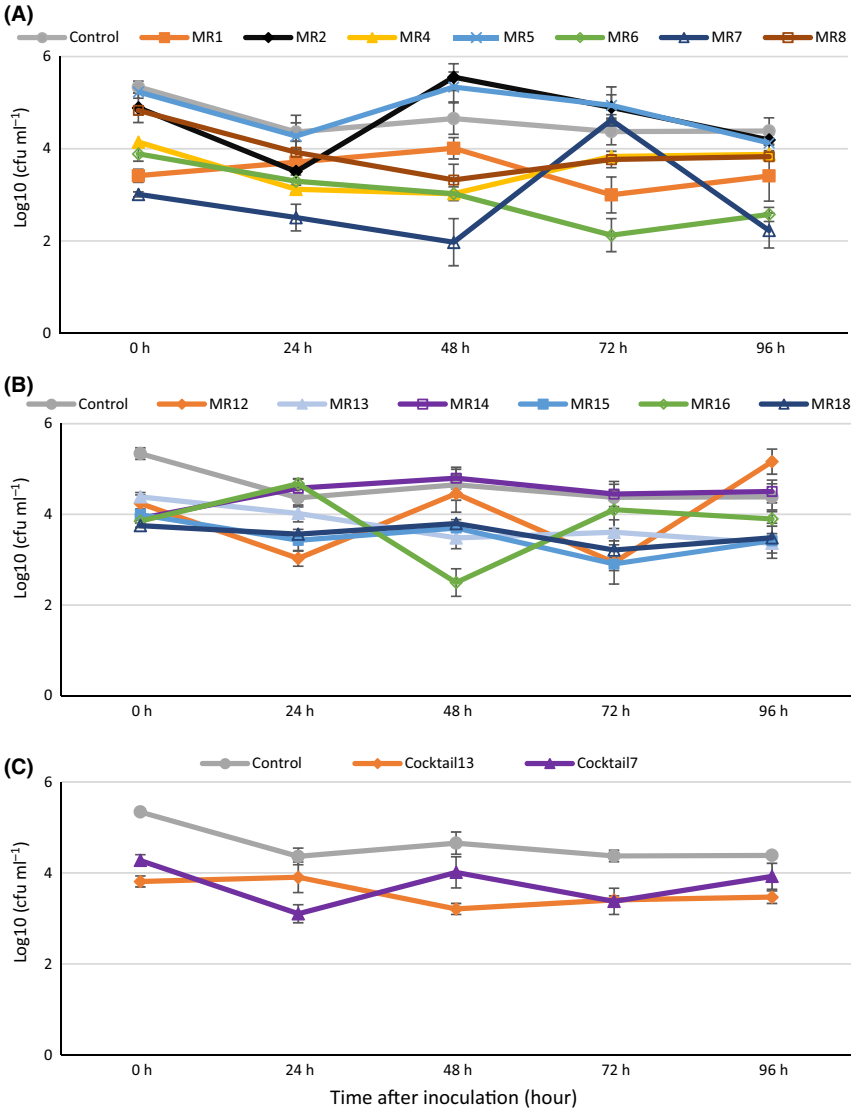


Fig. 5. Population dynamics of *P. syringae* pv. *syringae* (*Pss*) on bean leaves with and without phage treatments with single phage and two cocktails.
A. Control (*Pss*), MR1, MR2, MR4, MR5, MR6, MR7, MR.
B. Control (*Pss*), MR13, MR14, MR15, MR16, MR18.
C. Control (*Pss*), Cocktail13 and Cocktail7. Cocktail13 consists of all 13 phages and cocktail7 consists of MR4, MR6, MR8, MR13, MR14, MR15, MR16. Each value is a mean of nine replicates, and error bars represent the standard error of the mean (df: 1358, s.e.d: 0.4, phage*-time). The bacteria-only control (*Pss*) is the same in all graphs.

incubation, all 13 phages were stable at −20°C, 4°C and 20°C temperature, but only phage MR5, MR7, MR8 and MR12 survived at 27°C and none of the phage survived at 37°C. −20°C seems the ideal temperature for storage as bacterial resistance emergence was not observed after conducting plaque assays. These results support the use of phage as biological control agents as they maintain their stability and viability at a range of environmental temperatures. However, when applied in the plant environment, the phage should ideally be re-applied after two months to ensure the phage is able to infect and kill the bacterial pathogens particularly in hot summer months. Optimum growth temperature for *Pss* is between 5–30°C, which suggests that phages are capable of lysing bacteria at relevant temperatures (Latorre *et al.*, 2002). Rombouts *et al.* (2016) showed that phage KIL3 and KIL5 used against *Pseudomonas syringae* pv. *porri* were stable in phage buffer at a range of temperatures from 4°C to 37°C, but after 24 h of incubation at 50°C, a decrease in infectivity was noted for both phage KIL3 and KIL5. After freezing, all viable KIL3 phage were lost, while the titre of KIL5 decreased by three log10 units.

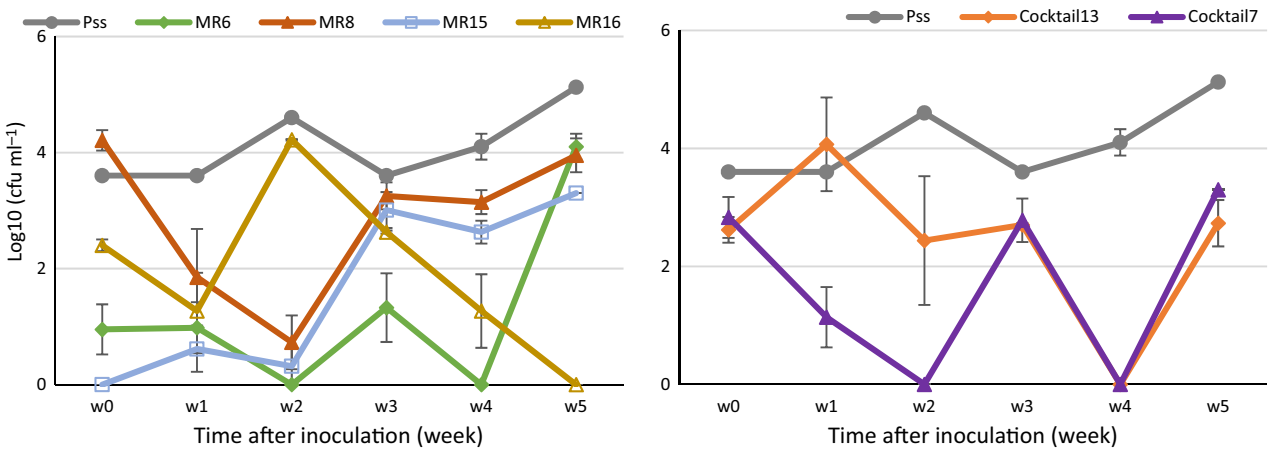


Fig. 6. Population dynamics of *P. syringae* pv. *syringae* (*Pss*) on cherry leaves with and without phage treatments with four single phage MR6, MR8, MR15 and MR16 and two cocktails. Cocktail13 consists of all 13 phages and cocktail7 consists of MR4, MR6, MR8, MR13, MR14, MR15, MR16. Each value is a mean of six replicates, and error bars represent the standard error of the mean (d.f: 604, s.e.d: 0.6, phage*time). The bacteria-only control (*Pss*) is the same in all graphs.

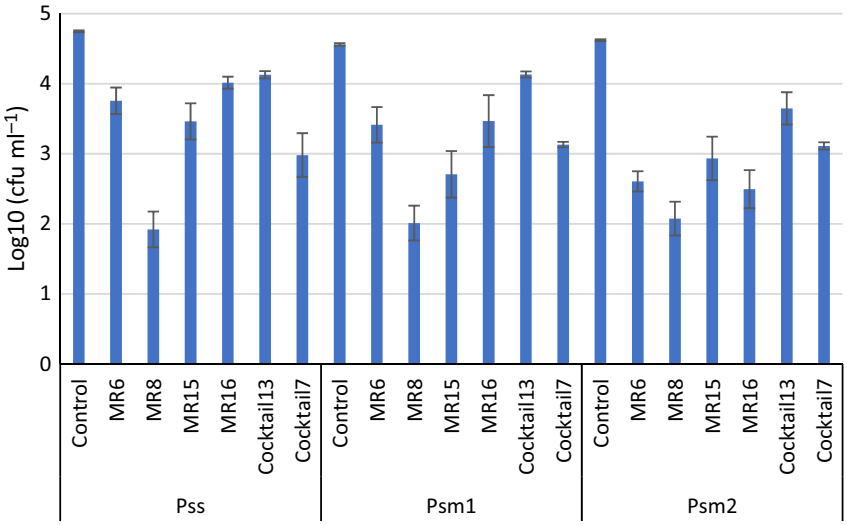


Fig. 7. Population dynamics of *Pseudomonas syringae* pv. *syringae* (*Pss*), *P. syringae* pv. *morsprunorum* race 1 (*Psm1*) and race 2 (*Psm2*) on cherry twigs with and without phage treatments with four single phage MR6, MR8, MR15 and MR16 and two cocktails. Cocktail13 consist of all 13 phages and cocktail7 consists of MR4, MR6, MR8, MR13, MR14, MR15, MR16. Each value is a mean of 30 replicates, and error bars represent the standard error of the mean (d.f: 562, s.e.d: 0.4).

Gašić *et al.* (2018) showed that at 20°C, KΦ1 phage concentration decreased more over time than the phages stored at 4°C, indicating that the lower temperature was more favourable for phage storage, as was also observed by Ritchie and Klos (1977). Thorne and Holt (1974) demonstrated that *Bacillus cereus* CP-51 phage was sensitive to low temperatures and survived better at room temperature, but the long-term storage of phage at ambient temperature is not generally recommended. These results suggest that phage with different host specificity and structures have dissimilar ability to survive the diverse temperatures. Even the slightest differences between two phages, not only result in differences observed in host range, but also in thermostability, underlining the importance of checking the temperature range at which phages are stable.

Results from the killing curve assay suggest that all 13 phages are effective at reducing bacterial populations. Generally, the higher the titre of the isolates, the faster they are at reducing the OD of *Pss, Psm1* and *Psm2.* A phage titre of 10^6 pfu ml^{-1} appears to be the best titre for phage preparation to reduce the development of bacterial resistance. High-titre phage inoculations may impart stronger selection pressures on the

bacteria, making the bacteria more likely to evolve resistance. Resistance evolution in response to phage has been noted in *Pseudomonas syringae* previously. Sistrom *et al.* (2015) studied *P. syringae* pv. *phaseoicola* resistance against phage Φ6. Resistance evolution was seen in the assay, almost exclusively at the highest titre of phage, at 10^{10} pfu ml^{-1}. However, when phages were applied in a phage cocktail, this resistance development was less likely to occur. Indeed, the possibility of resistance through lysogenic conversion was excluded, thereby confirming their suitability for phage biocontrol for this parameter (Gill and Hyman, 2009; Barbosa *et al.*, 2013; Rombouts *et al.*, 2016).

Phage genome sequencing subdivided the 13 phages into five distinct clades, which should be linked to taxonomic groups. The genomic structure of all phages was compared with sequences deposited in the GenBank database. We revealed that phage MR1 and MR2 have a high similarity to phage PPPL-1, a member of the *Autographivirinae* subfamily (Park *et al.*, 2018; Turner *et al.*, 2019). Within this family, PPPL-1 is predicted to belong to the genus of *Teseptimavirus*. As such, we posit that phages MR1 and MR2 may also be classified within this genus of phage and consideration should be given to assigning them to a new species within this genus according to the guidelines of the ICTV (Adriaenssens and Brister, 2017). Phage MR4 represents a new species of phage within the *Zindervirus* (*Autographivirinae* subfamily; Lee *et al.*, 2015). The collection of similar phages (MR5, MR6, MR7, MR8, MR12, MR16 and MR18) had low similarity to other phage genomes in the database, with the best hit being against *Pseudomonas* phage phi-2, a member of the *Autographivirinae* subfamily, belonging to the genus *Phikmvvirus*. Therefore, these phages might represent a new bacteriophage genus within the *Autographivirinae*. Phage MR14 had no sequence similarity to other phage genomes in the database, therefore being considered a novel phage that should be placed in a new taxonomic group. Phage MR13 and MR15 had low similarity to other phage genomes in the database, and therefore, they can be considered as novel phages, which should be placed in a new taxonomic group. Thus, our study has helped to expand knowledge of new phages and the diversity of phages infecting *P. syringae*.

All phages isolated in this study demonstrated a lytic infection cycle with the ability to target *Pss*, *Psm1* and *Psm2* isolates which cause canker on cherry. However, phage MR13 and MR15 genome sequence revealed one tRNA, with predicted proteins for a phage integrase as well as a putative repressor, indicating that both phages might be temperate phages, although, throughout all the experiments both phages produced clear plaques. Both MR13 and MR15 were isolated using *Psm1* strain 5244, and both are able to lyse most strains of *Pss*, *Psm1* and *Psm2* and also *P. syringae* pv. *tomato* strain DC3000 and *P. syringae* pv. *lachrymans* strain 789. Also, the killing curve data showed that both MR13 and MR15 were effective at reducing bacterial populations. These two phages were also able to reduce populations of *Pss* on bean and cherry leaves and twigs. Being potentially temperate might limit phage potential use in phage biocontrol as they can switch their life cycle to being lysogenic (Waldor, 1998; Boyd *et al.*, 2001; Merril *et al.*, 2003). However, they might be useful for *Pss*, *Psm1* and *Psm2* strain typing (Di Lallo *et al.*, 2014). Also, no toxin genes and virulence genes were identified within the phage genomes, confirming their potential use for phage-based biocontrol (Rombouts *et al.*, 2016).

To use phage as a biocontrol agent, bacteria-phage resistance needs to be investigated. *Pss*, *Psm1* and *Psm2* indeed developed some resistance to the phages. The identity of resistant bacteria, studied by rep-PCR, was similar to the original bacteria. Some of the phages were able to lyse the resistant bacteria. However, it is unclear what are the mechanisms of phage resistance, and thus, further analyses may be useful. Bacteria can develop resistance to phage in several ways (Petty *et al.*, 2006; Richter *et al.*, 2012). This can be via the modification of the cell surface, such as receptor mutation or receptor masking, injection blocking, restriction modification or abortive infection (Petty *et al.*, 2006; Richter *et al.*, 2012; Frampton *et al.*, 2014). More in-depth analyses of the BIMs genome may be useful in finding the mechanisms behind this resistance. A study indicated that BIMs of *Streptococcus thermophilus* had developed new CRISPR (clustered regularly interspaced short palindromic repeat) in specific regions of the genome. The short sequences were very similar to sequences of the phages used in the BIMs generation (Barrangou *et al.*, 2007). It is possible that the BIMs generated in this study have developed phage resistance in a similar manner, but the other ways cannot be excluded. Lysogeny in the resistant bacteria was tested and no plaque was observed, suggesting that the phages are unlikely to transfer genetic information to *Pss*, *Psm1* and *Psm2* (Frampton *et al.*, 2014). Although phage MR13 and MR15 are temperate phage, no plaque was produced by the resistant bacteria generated using these two phages.

The efficacy of the phage to lyse epiphytic bacteria in the plant environment *in vivo* was investigated on bean plants as a model system and on cherry leaves and twigs. *Pss* is the causal agent of bacterial brown spot of beans and is widely distributed epiphytically on bean leaves (Legard and Hunter, 1990), and thus, bean was chosen as an alternative host for the cherry pathogen.

No resistance symptoms were observed. Almost all phages were able to reduce the cfu per ml of *Pss* on bean leaves, almost immediately after application. The cocktail of all phages (cocktail13) and the cocktail7 (consisting of seven phages) were also able to reduce bacterial infection by almost 10-fold. Application of four of the phages individually, cocktail13 and cocktail7 on cherry leaves and twigs also showed that all phages were able to reduce bacterial population almost immediately after application and throughout the experiments. *Psm1* and *Psm2* did not colonize the leaves as well as Pss, it might be because *Psm1* and *Psm2* needs more time to establish on leaves environment. It could also be that Sweetheart variety is not a suitable host for *Psm1* and *Pms2*. Boulé *et al.* (2011) showed that infection of potted apple tree blossoms with the bacterial pathogen *Erwinia amylovora* could be reduced by phage ΦEa1337-26. A field trial of phage against *Xanthomonas* leaf blight of onion and tomato showed that weekly and biweekly applications of phage could reduce disease severity, a result which was comparable to treatments of weekly applications of copper–mancozeb. Phage application has previously been observed to be more effective than copper treatment in suppressing and reducing the disease severity (Obradovic *et al*., 2004; Lang *et al*., 2007). Rombouts *et al.* (2016) showed that phage therapy was able to reduce bacterial blight symptoms in leek caused by *P. syringae* pv. *porri*. However, applying phage efficiently in the field to ensure the persistence of phage in the phyllosphere must be further studied. High UV radiation can cause the destruction of phage particles; phage applied to tomato leaves in the early morning were undetectable the next day after UV irradiation (Iriarte *et al*., 2007). Several studies have sought to apply protective formulations to reduce loss of phage, such as skimmed milk powder (Balogh *et al*., 2010) which proved effective. Other methods explored involve applying phage along with attenuated bacterial strains to allow phage to replicate within the bacteria to a high concentration without infecting the plant (Iriarte *et al*., 2012). It may also be possible to force phage inside the plant tissue by way of an injection system, as the tree bark presents a physically impenetrable barrier to phage (James, 2015). This method is currently in use for other antimicrobial agents (Berger *et al*., 2015). Applying phage directly to the soil is also viable, and likely the most unobtrusive, scalable method of application (Iriarte *et al*., 2012). Therefore, to improve the efficacy of the phage in the field, phage persistence in the plant phyllosphere should be improved by the use of protective formulations, addition of non-pathogenic phage-propagating bacterial strains or by adapting timing and frequency of application as suggested previously (Jones *et al*., 2007; Balogh *et al*., 2010).

The phages isolated in this study are able to lyse *Pss*, *Psm1* and *Psm2* and have the potential to be used as biological control of bacterial canker. However, further study is required to investigate the ability of the phages to transfer genetic material from one bacterium to another. These phages should also be applied to cherry trees in the field to check their efficacy on all strains causing bacterial canker.

Experimental procedures

Bacterial strains and culture media

Pseudomonas syringae pv. syringae (*Pss*) strain 9097, *P. syringae* pv. *morsprunorum* race 1 (*Psm1*) strain 5244 and *P. syringae* pv. *morsprunorum* race 2 (*Psm2)* strain 5255 were used as host strains for isolation and propagation of phage isolates. Other *Pss, Psm1 and Psm2* strains were provided by NIAB EMR (Hulin *et al*., 2018a,b) which were collected within the UK from cherry and plum: *Pss* strains (9659, 9644, 9293, 9630, 9654), *Psm1* strains (9646, 9657, 5300, 9326, 9629), *Psm2* strains (5260, sc214, PSMR2 leaf, 9095), *Pseudomonas syringae* strains (9654 and 9643). Other bacteria used for analysis of host range included the following: *Pseudomonas syringae* pv. *tomato* DC3000, *P. syringae* pv. *lachrymans* 789, *P. marginalis* pv. *pastinaceae* 949, *P. cichorii* 907, *Citrobacter werkmanii* CwR94, *P. fluorescens* ATCC 17400, *P. fluorescens* F113, *P. fluorescens* PFR37, *P. fluorescens* Pf-5, *P. fluorescens* Pf-01, *P. fluorescens* WCS 365, *P. poae* PpR24 and *P. putida* PaW340. Kings B Medium (KB, 1 l distilled H_2O, 20 g protease peptone, 1.5 g K_2HPO_4, 1.5 g $MgSO_4.7H_2O$, 10 ml glycerol (plus 15 g l^{-1} agar for plates; King *et al*., 1954)) was used for culturing the bacterial strains. KB with 0.7% agar was used in the soft top agar overlay for the plaque assays. Phage buffer (10 ml 1 M Tris pH 7.5, 10 ml 1 M $MgSO_4$, 4 g NaCl, 1 ml 0.1 M $CaCl_2$, 980 ml double distilled (dd) H_2O) and phosphate-buffered saline (PBS, 1 L ddH_2O, 8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4{\cdot}2H_20$, 0.24 g KH_2HPO_4, pH 7.4) were used in phage dilution.

Phage sample collection from trees

Samples were taken from cherry trees in six cherry orchards in south east England in spring and summer of 2018: NIAB EMR trial plots (51°17′37.3″N 0°26′45.4″E, Penny, Vanda, Groton, Colney and wild cherry varieties), GH Dean commercial cherry orchard in Sittingbourne (51°20′01.6″N 0°47′02.9″E, Regina, Penny, Kordia, Vanda, Hertford and Lapins varieties), Brogdale National Fruit Collection (51°18′00.7″N 0°52′31.8″E, Stephens variety), an old cherry orchard in Sittingbourne (51°17′25.9″N 0°44′48.2″E, Penny, Sweetheart, Regina

and Lapins varieties) and an old cherry orchard and a young cherry orchard in Milstead (51°17′14.3″N 0°44′24.0″E, covered and uncovered, Penny and Sweetheart varieties). Soil samples were taken from the base of the tree, no more than a metre from the tree, at a depth of 10 cm, removing the surface soil. The soil corer used to take the soil samples was sterilized with ethanol between trees. Leaf samples were taken from the previous year's growth (measured using bud scale scars) on lower branches of the tree, with four leaves being taken per tree. Bark samples (1 cm × 1 cm) were taken using a sterile scalpel from outer and inner bark of the trees from only the NIAB EMR orchard. Samples were placed into sterile bags. All samples were stored at 4°C for later use.

Sample preparation, bacteriophage isolation and amplification

For phage isolation from soil samples, 2 g of soil was weighed and placed inside a 50 ml Falcon tube containing 20 ml PBS and three sterile tungsten beads (Qiagen, Manchester, UK). The tube was vortexed for one min and centrifuged at 4500 r.p.m. for 5 min. A 5 ml aliquot of resulting supernatant was filtered through a 0.22 μm filter (Millipore, Sigma-Aldrich, Gillingham, UK) to remove any bacteria. For leaf and bark samples, either 4 discs of leaf (approximately 1 cm in diameter) or one piece of bark (1 cm × 1 cm) were placed into a 50 ml Falcon tube containing 20 ml PBS and 3 sterile tungsten beads (Qiagen) and followed the above procedure. The resulting filtrate was stored at 4°C for use in plaque assays.

The optical density (OD) of overnight bacterial cultures were measured at 600 nm and adjusted to an optical density of 0.2 (≃ 10^8 bacterial cells per ml). 100 μl of the bacterial host and 100 μl of serially diluted environmental sample were added into soft top agar overlay and poured onto KB hard agar and incubated overnight at 27°C. Three successive single plaque isolations were performed to achieve pure phage isolates.

Phages were amplified by plating 10^6 plaque forming units (pfu) per plate with bacterial host on a soft agar overlay plate. After overnight incubation at 27°C, 5 ml PBS was added onto the plate and incubated at room temperature for one hour with agitation every 15 min. PBS was removed and filtered through a 0.22 μm filter to remove any bacteria. For bulking up, phage was enriched by adding 2 ml of bacterial cells at OD_{600} = 0.2 to a 50 ml Falcon tube containing 23 ml KB and incubated at 27°C shaking at 200 r.p.m. After 1 h, 1 ml phage sample, 50 mM $CaCl_2$ and 50 mM $MgCl_2$ were added to the bacteria and incubated shaking for 5 h. 250 μl chloroform (AnalaR, VMR, UK) was added and left to incubate for another hour. After incubation, the tube was removed and centrifuged at 4500 r.p.m. for 45 min at room temperature to pellet the bacterial cells. After centrifugation, the supernatant was removed, taking care not to disturb the bacterial pellet. The supernatant was filtered through a 0.22 μm filter into a fresh 50 ml Falcon tube, and polyethylene glycol (PEG) 8000 (Sigma-Aldrich) was added to the suspension to a final concentration of 8% w/v. After overnight incubation at 4°C, phage was precipitated by centrifugation (45 min, 4500 r.p.m. at 4°C) and the pellet was dissolved in 3 ml phage buffer. Phage were titered before storage at 4°C.

Host range assay

Phage host range was performed by spotting 5 μl of phage serially diluted suspensions (10^4–10^5 pfu ml^{-1}) on plates with a soft agar layer supplied with a specific bacterial isolate (OD_{600} = 0.2). The plates were incubated at 27°C for 24 h. After incubation, plates were checked for clearing where the spots of phage were applied.

Electron microscopy

To visualize phage using transmission electron microscopy (TEM), 400 μl of PEG precipitated (described above) samples (10^{10} pfu ml^{-1}) were centrifuged in ultra-centrifugal filter tube (30K, Thermo Fisher Scientific, Hemel Hempstead, UK) for 30 min at 15 000 r.p.m. at 4°C. Then, 10 μl was taken and spotted on a copper coated carbon–formvar TEM grid (300 mesh, Agaro) and left for 20 min to dry. Next, 10 μl of sterilized H_2O was spotted on the grid and left for 2 min. This was negatively stained with 10 μl of (1% w/v in distilled water) uranyl acetate (Thermo Fisher Scientific) for 2 min. Samples were analysed using a Phillips CM200 Transmission Electron Microscope (TEM) at accelerating voltage of 80 kV and photographs taken using an AMT camera system software.

Phage DNA extraction

A Phage DNA Isolation Kit (Norgen Biotek, Thorold, Canada) was used to extract phage DNA from a high-titre plate lysate (minimum of 10^8 pfu ml^{-1}) following the manufacturer's instructions. Extracted DNA was stored in a 1.5 ml ultracentrifuge tube at −20°C until needed. The DNA concentration and quality were measured using a NanoDrop 2000 (Thermo Fisher Scientific).

Random Amplification of Polymorphic DNA (RAPD)-PCR

RAPD-PCR was performed using DNA Primer P1 (5′-CCGCAGCCAA-3′, Eurofins, UK) at a concentration of 100 pmol μl^{-1}, with 10 μl GoTaq® Green Mastermix (Promega) and 8 μl sterilized water, all added to a 200 μl PCR

microtube. *Pss, Psm1 and Psm2* were used as controls alongside a negative control containing only sterilized water. Reaction conditions were followed as described by Gutierrez *et al*. (2011): four cycles at 94°C for 45 s, 30°C for 120 s and 72°C for 60 s; 26 cycles at 94°C for 5 s, 36°C for 30 s and 72°C for 30 s (the extension step increased by 1 s for every new cycle); a final step of 10 min at 75°C followed by 4°C for ∞. DNA amplicons were electrophoresed through a 1% (w/v, 0.5× Ambion® TBE buffer (Geneflow (UK), 10× solution contains 0.89 M Tris, 0.89 M Borate, 0.02 M EDTA)) agarose (Bioline, London, UK) gel.

Phage genome sequencing

The genomic DNA was sequenced using Illumina MiniSeq platform at the Laboratory of Gene Technology, KU Leuven (Belgium). A library was prepared using the Nextera Flex DNA Library Kit for each sample, tagged with a unique adapter sequence. The quality of each library preparation was controlled using an Agilent Bioanalyzer 2100. All library preps were equally pooled and sequenced using a MiniSeq Mid Output flowcell (300 cycles; 2*150 bp reads). After processing, the reads were trimmed with Trimmomatic (v0.36.5; Bolger *et al*., 2014). Genome assembly and annotation were done on the Galaxy (Afgan *et al*., 2018) and PATRIC (v3.6.2; Wattam *et al*., 2016) platforms using SPAdes (Bankevich *et al*., 2012) and RASTk (Brettin *et al*., 2015) respectively. Functional annotation was carried out by comparing translated ORFs in a BLASTp analysis (Altschul *et al*., 2005) against the non-redundant GenBank protein database (Kelley and Sternberg, 2009). When over 65% sequence similarity was present, that sequence was used as a reference to compare the sequenced genome with. Easyfig (Sullivan *et al*., 2011) was used to create linear comparison figures of multiple genomes and BLAST comparisons between multiple genomic regions.

Killing curve assay

To measure the effect of bacteriophage on bacterial growth, a killing curve assay was carried out. In a Greiner 96-well flat-bottomed plate, 100 μl of OD_{600} = 0.2 bacterial host in KB was aliquoted into each well of a row. Killing curves were generated at different multiplicity of infections (MOIs). 100 μl of each phage dilution in PBS was aliquoted to the corresponding well. This was repeated for each pathovar and corresponding phage strain. Two phage cocktails were also prepared: 'cocktail13' containing all phage strains and 'cocktail7' containing MR4, MR6, MR8, MR13, MR14, MR15, MR16. Sterile PBS was added to the bacterial broths as a negative control; and KB and PBS as a blank. The plate was loaded into a Magellan plate reader running Tecan software, measuring the optical density at 595 nm every 20 min for 19 h, with incubation at 27°C and shaking for 10 s before each reading.

Phage stability at different temperatures

To test the stability of the phages at different temperatures, phage suspensions ($10^{4–6}$ pfu ml^{-1}) were incubated at the following temperatures: −20°C, 4°C, 18°C, 27°C, 37°C in phage buffer. Infectivity of the phage was tested by incubating soft agar plates containing 100 μl of the bacterial host (OD_{600} = 0.2) with spots of different phage concentrations at 27°C. The pfu ml^{-1} of each phage was counted after 24 h, and the study was repeated every 7 days for 2 months and once after 6 months. Data were analysed using Genstat 18th edition (VSNI).

Bacterial resistance development to phage

Spontaneous mutants of *Pss*, that were resistant to phage infection (Bacteriophage-insensitive mutants – BIMs) were isolated as previously described in Frampton *et al.* (2014) and the frequency of bacterial resistance to phage was determined (McDonnell *et al*., 2018). Using the agar overlay method, 100 μl of phage and 100 μl of bacteria were plated at MOI 1, to lyse all bacteria on the plate. After 48 h of incubation at 27°C, emerging colonies were counted and four colonies were cultured and streaked to single colonies at least twice to ensure that a single strain was selected and to remove any phage particles (Frampton *et al*., 2014) and re-tested for resistance against phage infection. This was done in a second plaque assay using each phage against its corresponding single purified BIM isolate. To check, if each phage was able to lyse BIM generated using a different phage, 5 μl of each phage was spotted onto soft agar containing different BIMs.

Phage-resistant isolates were then analysed with repetitive DNA PCR-based genomic fingerprinting (rep-PCR) to confirm their *Pss*, *Psm1* and *Psm2* identity (Vicente and Roberts, 2007). Rep-PCR was performed in 20 μl volume containing: 10 μl GoTaq® Green Mastermix (Promega), 2 μl of dimethylsulphoxide (DMSO; Sigma-Aldrich), 1 μl of 3.2 lg bovine serum albumin (BSA; Sigma-Aldrich) and 1 μl 40 pmol of each primer REP, ERIC and BOX (Eurofins, UK). DNA of resistant bacteria was prepared as follow: 1 ml of overnight culture was centrifuged at 13 000 r.p.m. for 3 min. The supernatant was discarded, and the pellet was re-suspended in 200 μl TE buffer (1 M Tris-HCL, 0.5 M EDTA pH 8). After vortexing, the cell suspension was heated at 100°C for 10 min on a heating block. The tube was then chilled on ice for 10 min followed by second

centrifugation. The supernatant was transferred to a new tube and 2 μl used in a PCR reaction (Vicente and Roberts, 2007).

PCR conditions were as follows: one initial cycle at 95°C for 2 min; 30 cycles of denaturation at 94°C for 3 s and 92°C for 30 s, annealing at 40, 52 or 53°C for 1 min with REP, ERIC and BOX primers, respectively, extension at 65°C for 8 min; single final extension at 65°C for 8 min and then held at 4°C (Vicente and Roberts, 2007). PCR products were separated by gel electrophoresis on a 2% agarose gel for 2 h at 60 V.

Lysogeny in these resistant bacteria was tested. The supernatant from overnight cultures of resistant bacteria were collected and tested for the spontaneous release of phage particles by spotting 5 μl drops onto soft agar containing the different bacterial hosts (*Pss*, *Psm1* and *Psm2*) and observing for plaques (Rombouts *et al.*, 2016).

Bioassay

The efficacy of the phages (and phage cocktails) at reducing disease progression was evaluated *in vivo* using bean plants (*Phaseolus vulgaris*), a known host of *P. syringae*. Three bean seeds (Cobra Climbing French variety) were planted in 5-inch pots containing John Innes compost (no. 1) and incubated at 22°C with each pot having five replicates, per sample, arranged randomly. *Pss* (OD_{600} = 0.2) was prepared in PBS and phage suspension was PEG precipitated in phage buffer (10^6 pfu ml^{-1}) and placed into mist sprayers (transparent fine mist spray, Amazon, UK). Beans were grown for approximately ten days until bifoliolate stage. All leaves were first sprayed with bacterial host and left to dry for 30 min, three leaves were randomly collected from five pots (control, 0 h). The rest of the samples were sprayed with the phage and left to dry for 30 min and three leaves were collected immediately (0 h). Pots were placed in a plastic grow cover (Verve, B&Q, UK), to maintain humidity. Leaf samples were collected every 24 h for 5 days. From each collected leaf, four discs (1 cm in diameter) were removed and placed into a 2 ml tube containing 1 ml PBS with two tungsten beads and homogenized at a speed of 6 m s^{-1} for 10 s. Serial dilutions were prepared, and 3 × 25 μl of neat, 10^{-1}, 10^{-2}, 10^{-3} serially diluted samples were spotted onto KB agar plates containing 100 μg ml^{-1} Cycloheximide (Sigma-Aldrich). Plates were incubated at 27°C, and the number of colony forming units (cfu ml^{-1}) was recorded after 24 h.

Cherry leaf inoculation was conducted on two-year-old cherry cultivar Sweetheart on rootstock Gisela 5 (dwarf) in 12 l pot (Ornamental Trees, UK). *Pss*, *Psm1* and *Psm2* suspensions of ≃ 10^8 cfu ml^{-1} in PBS and phage suspension (10^6 pfu ml^{-1}) were used for inoculation. Leaves were fully covered with bacteria using hand sprayers and left to dry for a day. Phage inoculation was done the day after. PBS, bacteria and phage only treatments were used as control. Each branch of a tree was used as a treatment. Two trees were used per phage inoculation. Trees were incubated in a controlled environmental chamber (Weiss-Technik, UK) at day/night temperature 25/15°C, 85% humidity and 14/10 h day/night length. Leaf samples were collected one day after phage inoculation (week 0), and thereafter samples were collected every week for 5 weeks. The above procedure for beans was performed to calculate cfu ml^{-1}.

Twig inoculations were performed as previously described by Hulin *et al.* (2018a,b). Bacterial suspensions of 10^8 cfu ml^{-1} were used for inoculations through wounds. A sterile scalpel was used to cut a shallow wound at an angle into the twigs, and 100 μl of bacterial inoculum was pipetted into the wound. The next day, each wound was inoculated with 100 μl of phage suspension (10^6 pfu ml^{-1}). The inoculation sites were covered with Parafilm (Sigma-Aldrich). Five inoculations were performed on the same twig. PBS, bacterial and phage only treatments were used as control. Woody samples were collected 40 days after inoculation from the infection sites and analysed for the presence of bacteria by plate count of cfu in the samples. 0.5 g of woody tissue was added to 1 ml of sterile PBS and homogenized, and the above procedure was performed to calculate cfu ml^{-1}. Data were analysed using Genstat 18th edition (VSNI).

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Conflict of interest

The authors declare that they have no conflict of interest.

Authors contributions

M.R. and R.W.J. conceived and contributed to experimental design. M.R. and S.R.R. carried out experimental work. L.F., B.J.Q., R.C. and G.W.S. contributed to experimental work. M.R., D.H., J.W. and R.L. contributed to genome sequencing. M.R. performed statistical analysis and wrote the manuscript. All authors contributed to interpretation of results, read and approved the final manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Host range, source and location of 70 phages isolated in this study.

Fig S1. RAPD-PCR profile image of 18 phages characterised in this study.

Fig S2. *In vitro* killing curve displaying the effect of phages used in this study on *Pseudomonas syringae* pv. *syringae (Pss)* population growth.

Fig S3. Temperature stability of phages used in this study.

Fig S4. Rep-PCR profile image of bacteria resisted to phage.

